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Identification and quantification of trehalose in vesicular-arbuscular mycorrhizal fungi by *in vivo* ^{13}C NMR and HPLC analyses*

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SUMMARY

Natural abundance *in vivo* ^{13}C NMR spectra were obtained from spores of three species of VA mycorrhizal fungi: *Glomus intraradix* Schenck & Smith, *Glomus etunicatum* Becker & Gerdemann and *Gigaspora margarita* Becker & Hall. The presence of trehalose was established in the spectra of *Gl. etunicatum* and *Gi. margarita*. HPLC analyses supported these observations and indicated that *Gl. intraradix* spores also contained small amounts of trehalose, not readily detectable by ^{13}C NMR. Trehalose constituted between 0·06% and 1·6% (w/w) of the spore dry weight, depending on the species. Only trace amounts of other sugars, including glycerol, were detected. An *in vivo* NMR time course experiment indicated that trehalose was readily utilized during spore germination.

Key words: Vesicular-arbuscular mycorrhiza, trehalose, spore, ^{13}C NMR, HPLC.

INTRODUCTION

The biotrophic endophyte of mutualistic and pathogenic plant-fungus associations converts carbohydrates it receives from the host (mainly sucrose) into fungus-specific carbohydrates such as polyols and trehalose (Smith, Muscatine & Lewis, 1969). Glycogen represents a form of ultimate storage (Kinden & Brown, 1975; Holley & Peterson, 1979; Gianinazzi-Pearson *et al.*, 1981). It has been proposed that these conversions maintain a concentration gradient and act as a one way 'biochemical valve' for further movement of sucrose (Lewis & Harley, 1965; Smith *et al.*, 1969; Harley & Smith, 1983).

Vesicular-arbuscular [VA] mycorrhizas represent the most widely distributed plant-fungus association. The fungal symbiont of this association, like other biotrophic endophytes, requires host photosynthates for growth. Attempts to demonstrate the occurrence of 'biochemical valves' in this symbiosis have been made but neither polyols nor trehalose have been found in VA mycorrhizal fungi (Hayman, 1974; Hepper & Mosse, 1972; Bevege, Bowen & Skinner, 1975), except for a report of preliminary results (Amijee & Stribley, 1987) which mentions the possible presence of trehalose and another unknown sugar in *Glomus mosseae* and *Glomus caledonium*.

In this paper, we report the analyses of carbohydrate contents of spores of three species of VA mycorrhizal fungi. Natural abundance *in vivo* ^{13}C nuclear magnetic resonance [NMR] analyses were made on large samples of spores and verified with HPLC analyses of spore extracts. These analyses were performed before and after germination for one of the three species studied.

MATERIALS AND METHODS

Spores

Chlamydospores of *Glomus etunicatum* Becker & Gerdemann along with a mixture of equal quantity (Tim Wood, personal communication) of intra- and extraradical spores of *Glomus intraradix* Schenck & Smith were purchased from Native Plant Industries (Salt Lake City, UT). Exclusively soil-borne spores of the latter species were also obtained from Les Tourbières Premier (Rivière du Loup, Québec,

* Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

Abbreviations: TMS, Tetramethylsilane; VA, vesicular-arbuscular; N.O.E., Nuclear Overhauser Effect.

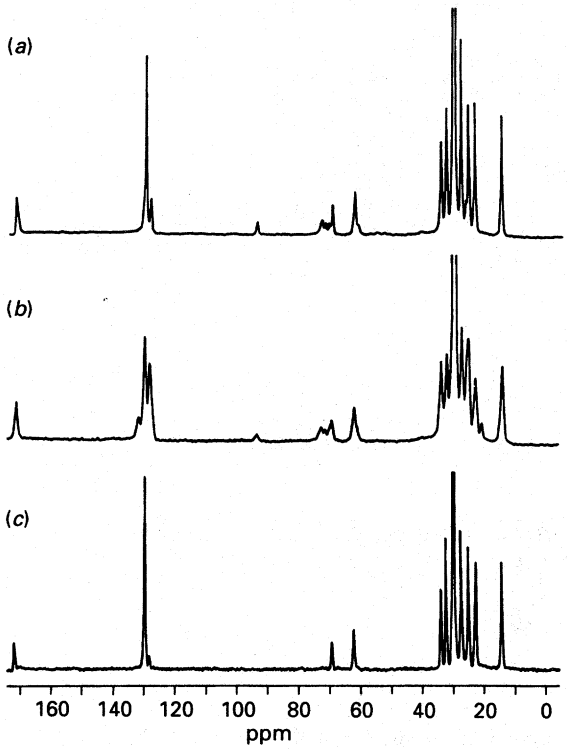

Figure 1. 100·4 MHz proton decoupled ^{13}C spectra of intact spores of: (*a*) *Gigaspora margarita*, (*b*) *Glomus etunicatum* and (*c*) *Glomus intraradix*. Each spectrum was acquired at 30 °C with a 20 KHz spectral width, 16K data points, a 70° pulse angle, repetition time of 2·2 s and exponential line broadening of 8 Hz. Spectra (*a*) and (*c*) were obtained with 3200 scans and spectrum (*b*) was obtained with 3782 scans. Chemical shifts are referenced to the terminal CH_3 group of the fatty acid at 14·1 ppm relative to external TMS.

Canada). Azygospores of *Gigaspora margarita* Becker & Hall were produced at C.R.B.F. (Laval University, Québec, Canada) as already described (Bécard & Fortin, 1988).

The spores were collected by wet sieving and further purified by several centrifugations in 40% (w/v) sucrose followed by a density gradient centrifugation in Renografin-60 (Furlan, Bartschi & Fortin, 1980). Approximately 5×10^4, 5×10^5 and 3×10^5 spores were collected for *Gi. margarita*, *Gl. intraradix* and *Gl. etunicatum*, respectively. They were surface sterilized as described by Bécard & Fortin (1988).

NMR spectroscopy

Proton decoupled ^{13}C spectra of intact spores were obtained on a 9·3 T JEOL GX-400 NMR spectrometer operating at 100·4 MHz. Spores, suspended in 1:1 H_2O/D_2O, were layered on top of a 15 mm plug of solid agar substitute [0·8%] (Gel-Gro, ICN Biochemicals, Cleveland, OH, USA) in the bottom of a 10 mm NMR tube so the spores filled the 1 ml volume of the magnetic coil more efficiently. Spectra (3200–33 900 scans) were obtained at 30 °C with 20 KHz or 25 KHz spectral widths, 16K data points, 2·2 s delays and a 70° pulse angle. Exponential broadening was 5·0 or 8·0 Hz. No spinning of the samples was necessary since the linewidths were of the order of 30–40 Hz wide. Quantitative spectra were obtained by suppressing the N.O.E. with an inverse gated decoupling using a repetition time of 5 s. Spectral integration was accomplished with stepped integrals as well as with cut-out and weighing procedures.

Spore germination

After being analyzed by ^{13}C NMR spectroscopy, spores of *Gl. etunicatum* were axenically transferred to two Petri plates and incubated in distilled water, free of D_2O, at 30 °C in a CO_2 incubator (2% CO_2) for five days. More than 70% of the spores germinated under these conditions. The spores were reintroduced into the NMR tube for further analysis after checking for bacterial and fungal contamination.

Isolation of carbohydrates and HPLC analysis

Following *in vivo* NMR analyses, spores were freeze-dried, weighed and stored in a freezer. An additional sample of *Gl. etunicatum* was analyzed at the pre-germination stage. Spores were ground in a mortar with methanol-water (70/30 v/v). The extracts were then centrifuged at 18×10^3 ***g*** for 20 min and the supernatants from three successive washes were pooled. The methanol was removed at 40 °C by evaporation under reduced pressure. The remaining aqueous phase was extracted twice in a separatory funnel with two volumes of methylene chloride and once with two volumes of hexane to remove lipid. All ionic compounds then were removed by passing the aqueous phases through a 10 ml column of Amberlite MB-3 resin (16–50 mesh, Sigma) at 1·0 ml min^{-1}. The column eluents were freeze-dried in tared flasks to yield fluffy white carbohydrate fractions. One percent solutions of these fractions were prepared for HPLC analysis by dissolving the powder first in water and then in an equal volume of acetonitrile, so that 200 μg 20 μl^{-1} were injected. HPLC separations were carried out on a Spectra-Physics (San Jose, CA, USA) system equipped with a Waters (Milford, MA, USA) refractive index detector, a Rainin (Woburn, MA, USA) Dynamax 60A NH_2 analytical column (4·6 × 250 mm) and a 20 μl loop injector. The carbohydrates were separated by isocratic elution of the NH_2 column with acetonitrile:water (65/35, v/v) at 1·0 ml min^{-1}. Quantitation of carbohydrates in the spore extracts was based on comparisons of peak heights (Spectra-Physics Integrator-Recorder) with carbohydrates in a standard mixture (glycerol, fructose, glucose, sucrose and trehalose at 24·4, 12·6, 33·0, 10·4 and 53·7 μg 20 μl^{-1}, respectively). Fructose, glucose, sucrose, α,α-

trehalose and glycerol were purchased from Sigma (St. Louis, MO, USA), as were the enzymes, trehalase and glucose oxidase.

RESULTS

Figure 1 shows the *in vivo*, natural abundance, proton decoupled ^{13}C NMR spectra of (*a*) *Gi. margarita*, (*b*) *Gl. etunicatum* and (*c*) *Gl. intraradix*. Only one spectrum for the last species is shown because there was no difference between the two samples of different origin. The resonances from 14 ppm to approximately 34 ppm represent fatty acid chain carbons; the carbohydrate region is from 60 to 95 ppm. Various unsaturated carbon resonances and carboxyl carbon resonances occur at approximately 130 and 172 ppm, respectively. An expansion of the carbohydrate region (Fig. 2*a*) of the spectrum shown in Figure 1(*a*) reveals the C1,3 and C2 triglyceride-derived glycerol resonances at 61·84 and 69·00 ppm, respectively, overlapping the trehalose resonances. A solution of pure α,α-trehalose (Fig. 2*b*) confirms the identity of the sugar in the *in vivo* spectrum (Fig. 2*a*) [cf. our earlier assignment (Pfeffer, Valentine & Parrish, 1979)].

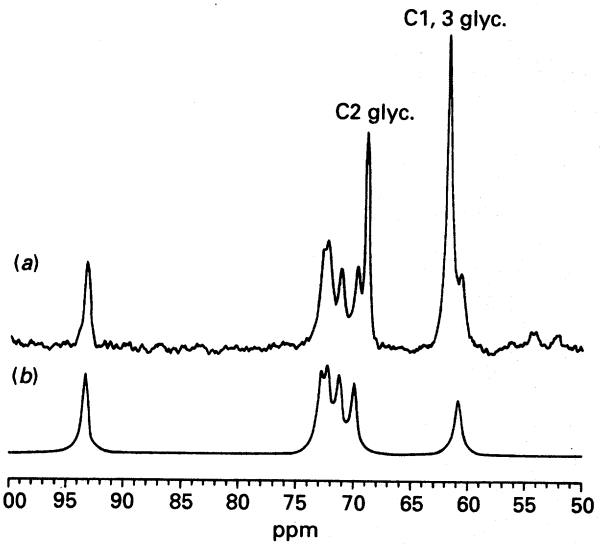

Figure 2. 100·4 MHz proton decoupled ^{13}C spectra of: (*a*) *Gigaspora margarita* spores showing the carbohydrate region, (acquisition parameters as in Figure 1, except the exponential linebroadening of 15 Hz was used) and (*b*) authentic α,α-trehalose 10 mg ml^{-1} [acquisition parameters as in (*a*) except only 128 scans were collected and 40 Hz linebroadening was used to simulate the broader lines of the *in vivo* spectrum (*a*)]. glyc = glycerol.

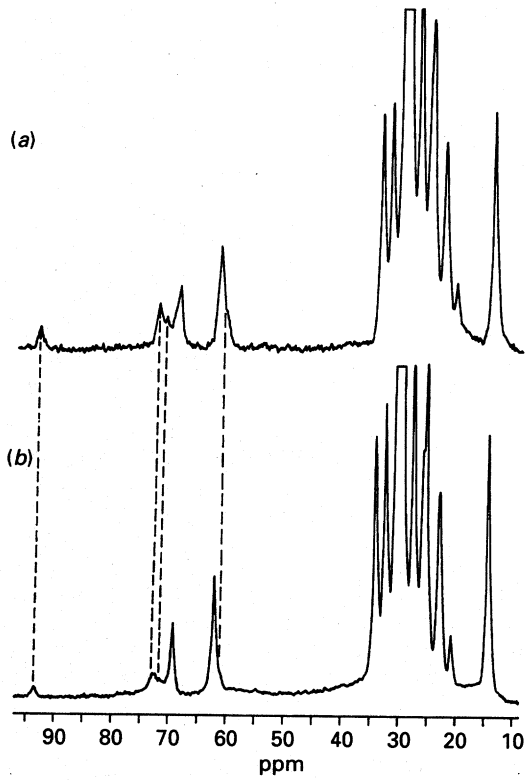

Figure 3. 100·4 MHz proton decoupled ^{13}C spectra of: (*a*) *Glomus etunicatum* spores prior to germination at 30 °C, [acquisition parameters as in Figure 1 (*b*), 3782 scans] and (*b*) same spores after 5 days of germination at 30 °C, 33937 scans. A greater number of scans was necessary for this spectrum because of the diminished density of spore sample owing to dilution with hyphal tissue.

We were able to obtain values for the carbon distribution between trehalose and lipid (Martin, Canet & Marchal, 1984a), the distribution of lipids between triglycerides and free fatty acids, and the degree of unsaturation of fatty acids (Martin *et al.*, 1984b) based on the N.O.E. suppressed ^{13}C spectra of each species (Table 1). *In vivo* ^{13}C NMR spectra can only account for the mobile lipids and cannot give a detailed analysis of all the lipid components as recently demonstrated by GLC (Jabaji-Hare, 1988).

Table 1. *^{13}C NMR analysis of dormant spores*

Fungal species	Carbon distribution (mole %)		Lipid distribution (mole %)		Fatty acid distribution (%)			
						Unsaturated		
	Trehalose	Lipids	Triglycerides	Free F.A.*	Saturated	Mono-	Di-	Tri-
Gigaspora margarita	5·0	95·0	54·0	46·0	31·7	63·5	n.d.*	4·8
Glomus intraradix	n.d.	100	75·0	25·0	33·3	65·0	n.d.	1·7
Glomus etunicatum	4·7	95·3	83·7	16·3	25·8	45·9	16·1	12·2

All values are ±10%.
* F.A. = fatty acids; n.d. = not detected.

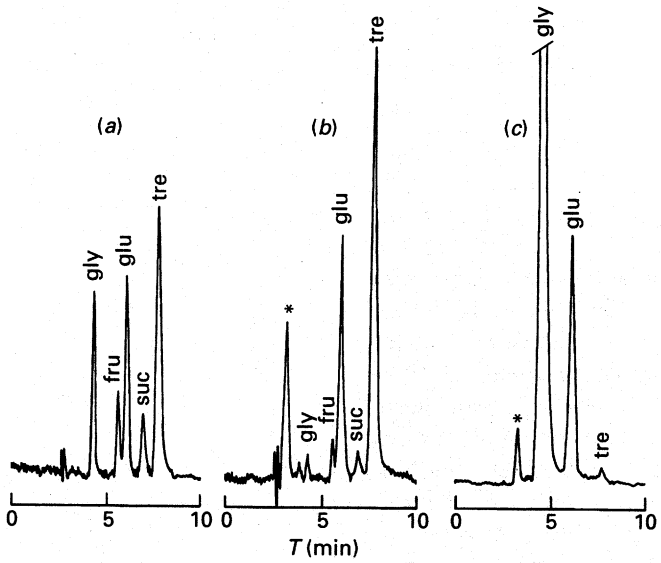

Figure 4. High performance liquid chromatograms (conditions in Materials and Methods) of (*a*) standard mixture of glycerol (gly), fructose (fru), glucose (glu), sucrose (suc) and trehalose (tre); (*b*) extract of *Gigaspora margarita* spores and (*c*) extract of *Gigaspora margarita* spores treated with trehalase, the preparation of which contains glycerol as a stabilizer. Attenuation 32X for (*a*) and (*b*); 64X for (*c*). Remaining traces of methanol (*) used as the extractant are visible in chromatograms (*b*) and (*c*).

Table 2. *Carbohydrate content of spores (as % of dry weight) for three species of VA mycorrhizal fungi, based on HPLC analyses*

	Gigaspora margarita	*Glomus intraradix*	*Glomus etunicatum*	
	(Before germination)	(Before germination)	(Before germination)	(After germination)
Trehalose	1·130	0·060	1·600	0·670
Glucose	0·470	TR	0·029	0·014
Glycerol	TR*	TR	TR	TR
Fructose	TR	n.d.*	n.d.	n.d.
Sucrose	TR	n.d.	n.d.	n.d.

* TR = trace; n.d. = not detected.

Figure 3 shows the spectrum of the carbohydrate and fatty acid regions (saturated carbons) before (*a*) and after (*b*) germination of spores of *Gl. etunicatum*. The trehalose diminished by 50% during germination relative to fatty acid and glycerol. No significant change in fatty acid composition or triglyceride content nor the appearance of other carbohydrates was noted.

Glycerol, fructose, glucose, sucrose and trehalose in a standard mixture were rapidly resolved on the NH_2 HPLC column (Fig. 4*a*). The chromatogram of the extract of *Gi. margarita* (Fig. 4*b*) is given as an example of carbohydrate analysis of spore extracts. The peak at 6·18 min was glucose and not mannitol (which has a similar retention time) since the peak collapsed when glucose oxidase was added. Since glucose was not observable in the ^{13}C *in vivo* spectrum of the *Gi. margarita* spores, we suspect that glucose had been produced from the breakdown of some polymeric form during the extraction procedure. Extracts from the three species contained different amounts of these compounds (Table 2). No difference was found between the two samples of *Gl. intraradix* spores. The presence of trehalose in the extracts was confirmed when treatment with trehalase resulted in an increase in glucose at the expense of trehalose (Fig. 4*c*). The large glycerol peak was due to its presence as a stabilizer in the trehalase preparation. The germination of *Gl. etunicatum* spores was accompanied by a 58% decrease in trehalose (Table 2), a finding in good agreement with the 50% value from the NMR time course analysis.

DISCUSSION

These results clearly establish for the first time that trehalose is present in VA mycorrhizal fungi. This finding was predicted by Lewis (1975) because trehalose is a major component of many species of coenocytic fungi, especially the Mucorales to which VA mycorrhizal fungi belong. Presence of free

glycerol in the three species studied is also in agreement with the finding that this polyol is present in many zygomycetes (Pfyffer, Pfyffer & Rast, 1986). However, this observation, based on HPLC analyses, requires further confirmation since glycerol might have been formed during the extraction procedure.

Trehalose had not been detected in VA mycorrhizal fungi in past investigations, possibly because those investigations were made on mycorrhizal roots with a relatively low amount of fungal tissue. In contrast, large numbers of spores enabled us clearly to detect trehalose. We conclude that this disaccharide is the major storage carbohydrate in VA mycorrhizal fungi. Therefore, VA mycorrhizas should no longer be considered an exceptional plant-fungus association with regard to the 'biochemical valve' hypothesis (Harley & Smith, 1983). It remains to be established where and when the irreversible conversion of host photosynthates to trehalose occurs in the fungal life cycle. The relatively smaller concentration of trehalose found in *Gl. intraradix* could not be explained by the presence of intraradical spores since the same result was obtained with a sample of pure extraradical spores. This is indirect evidence that trehalose can also be produced by the intraradical phase of the fungus. Recently, trehalose also has been reported in roots colonized with either of two *Glomus* species, *Gl. versiforme* and *Gl. mosseae*, as well as in their sporocarps (Schubert, Wyss & Wiemken, 1990).

The decrease of trehalose (50–58 %) after five days of incubation of *Gl. etunicatum* spores indicates that this metabolite is used in an early stage of germination and may be a readily available source of energy to prime metabolic processes in the spores, as it is for spores of many other fungi (Thevelein, den Hollander & Shulman, 1984). Subsequent hyphal growth generally starts on the fourth day, and, more likely, is supported by lipid catabolism.

A second possible function exists for trehalose in VA mycorrhizal fungi. Trehalose stabilizes membranes during freezing/dehydration stress (Crowe, *et al.*, 1986) and the cytoplasm of yeast during stress (Wiemken, 1990), and imparts a measure of cryoprotection to *Gi. margarita* spores (Douds & Schenck, 1990). However, more than 1 g of trehalose was needed to stabilize fully 1 g of liposomes *in vitro* and organisms which rely upon biochemical adaptations to survive dehydration typically have 15–20 % of their dry weight as trehalose (Crowe, *et al.*, 1986). We found trehalose to be in the range of 1 % dry weight of VA mycorrhizal fungus spores, perhaps too little to serve primarily to stabilize membranes.

This paper reports the first *in vivo* natural abundance ^{13}C NMR experiments on VA mycorrhizal fungi and gives an example of a time course experiment which compares spores at two different physiological stages. Similar experiments have been carried out using ^{31}P NMR as a metabolic probe (data not shown). A study of the metabolism involved in spore germination utilizing a ^{13}C enriched CO_2 atmosphere will be reported in the future.

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